Supplementary Materials for

Spatiotemporal microbial evolution on antibiotic landscapes

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Other Supplementary Materials for this manuscript includes the following:

Materials and Methods

Strains

The Δ*lacA* strain from the Keio collection of *E. coli* K-12 BW25113 knockout variants(*34*) was inoculated in the center of 0.28% agar LB Petri dishes. After overnight growth, cells were selected from the periphery of the spreading colony. This was repeated five additional times. Cells were then grown overnight in LB and frozen in glycerol stocks at -80C. The MEGA-plate was inoculated with between 30 and 100µl of this stock after the top agar had cooled and gelled. Knockout mutant assays were performed on the corresponding strain from the Keio collection.

MEGA-plate Construction

The full-scale MEGA-plates were built using a 2'x4'x3/16" clear polycarbonate sheet, with bars of ½"x2" clear polycarbonate walls and ½"-high interior divisions laser-cut from ⅛" acrylic. All materials were obtained from McMaster-Carr. While either acrylic or polycarbonate can be used for both purposes, we have found that an top sheet and a polycarbonate base and walls provide the best combination of optical clarity for imaging and resistance to thermal flexing. Components were welded using dichloromethane, and leaks were sealed with DAP all-purpose 100% silicone adhesive sealant. The quarter-size MEGA-plates were built using a 1'x2'x3/16" clear polycarbonate baseplate with walls made from bars of ½"x2" polycarbonate and interior divisions as above. The MEGA-plates were decontaminated before and after experiments by filling them with ~10% bleach overnight.

MEGA-plate Setup

The base agar was composed of 2% w/v BD Bacto Agar (214030), and one LB-Lennox capsule (RPI #L24065) per liter.

The risk of contamination was minimized by autoclaving the agar for two 40-minute cycles (instead of one). Further, to suppress contaminants, a supplement of 5ml of 6mg/ml Kanamycin (Sigma #K1876, final concentration 30µg/ml) and 20mg/ml Cycloheximide (Sigma #C7698 final concentration 100µg/ml) was added to each liter of media.

Trimethoprim (Sigma #T7883) stock at 100mg/ml in DMSO was added (between 10µl and 10ml) to stated concentrations. 4ml of autoclaved India Ink (Higgins #44204) was added to each liter for contrast. The soft agar overlay was composed of 0.28% w/v Agar with LB, Kanamycin, and Cycloheximide as above. Once the nine liters of black agar set, three additional liters of black agar without trimethoprim were added on top and allowed to solidify. The addition of a thin layer of solid agar between the bottom and overlay decreases inhomogeneity of growth between sections by physically leveling the interface Once these solidified, the two liters of soft agar were poured on top.

The experiments presented here were all poured open on a laboratory bench, and covered as soon as the soft overlay agar was poured. Prompt covering following this addition is crucial, otherwise uneven setting can occur, yielding uneven agar densities in the swim media. After the agar has set, briefly opening the cover can decrease condensation, which in turn decreases the presence of liquid along the edges through which the bacteria can more quickly migrate.

Temperature Control

For all experiments, except the one shown in fig. S5, space heaters (Boston, 25964) on each of the short sides were used to maintain both room temperature and to prevent condensation on the lid. The two on each side were alternatively turned on every 300 seconds by an Arduino Uno R3-controlled relay board (Winford Engineering, RLY104-5V-FT). While the room could not be temperature controlled, measurement of the temperature below multiple points in the MEGA-plate showed a consistent temperature of 27-29°C.

In fig. S5, the experiment was performed in a temperature-controlled room at 30°C, with a heated glass cover.

Imaging

The relative speed of spreading in the MEGA-plate, empirically observed at ~40mm/hour, implies that time-lapse photography at 10-minute intervals will yield sufficient resolution for evolutionary dynamics. The addition of india ink in the base agar and white-light imaging with desk-lamps at an oblique angle results in a high-contrast dark-field image across the plate.

The time-lapse photography was taken with a Nikon D5100 controlled by NKRemote (Breeze Systems), with the exception of Movie S2, which was imaged with a Canon Powershot G10. Images were aligned, cropped, white-balanced, and linearly (and uniformly per-stack) contrast-enhanced in MATLAB (Mathworks). Movies were assembled from the processed images in Picasa 3 (Google).

Images for fig. S5 were captured with a Canon T3i, and were linearly contrast-enhanced uniformly in each color channel. All enhancements are uniform across all regions of all images in the same figure.

Four-step MEGA-plate Setup

We set up the MEGA-plate in nine equal-width (13.3cm) bands running widthwise across the plate (Fig. 1A, fig. S1). We put no antibiotics in the outermost bands. In the remaining solid bands, going inwards from both sides, we put an exponential gradient of drug (1μg/ml, 10μg/ml, 100μg/ml, 1mg/ml for Trimethoprim, 500ng/ml, 5μg/ml, 50μg/ml, 50μg/ml of Ciprofloxacin). Once the drug had vertically equilibrated with the additional, drug-free agar layers, the final antibiotic concentrations were 56% of the bottom agar. We inoculated *E. coli* on either side and took an image of the MEGA-plate from above every 10 minutes for the next 12 days.

Intermediate-step MEGA-plate Setup

For TMP we set up the MEGA-plate in nine equal-width bands as above, with concentrations (1mg/ml, 100μg/ml, 0, 10 μg/ml, 1mg/ml, 1 μg/ml, 0, 0, 1mg/ml). Similarly the final concentrations were 56% of the base concentration. We inoculated the first and second sections lacking antibiotic with *E. coli* and imaged as above. For CPR, we constructed four miniature MEGA-plates measuring 30.5 x 61cm with longitudinally oriented bands. The center band was 13.3cm, as above, and the outer two were of equal width. All of the left bands had no antibiotic, all of the right ones had 50μg/ml, the intermediate band had 0, 50ng/ml, 500ng/ml, or 5μg/ml depending on the plate. We inoculated all of the regions without antibiotic and imaged as above.

Sample Phenotyping

We sampled between three and five single-colony isolates from each marked point. All isolates were assayed for MIC to the drug from which they were picked, and a representative was chosen by consensus phenotype. MICs were measured by pinning onto LB agar plates containing 0, 0.56μg/ml, 1.77μg/ml, 5.6μg/ml, 17.7μg/ml, 56μg/ml, 177μg/ml, 560μg/ml, and 1.77mg/ml for TMP and for 0, 0.28μg/ml, 0.889μg/ml, 2.8μg/ml, 8.89μg/ml, 28μg/ml, 88.9μg/ml, 280μg/ml, and 889mg/ml CPR. Growth was assessed after 24 hours of incubation at 30C. Strain MIC test plates were imaged with a Canon T3i under translumination and were analyzed in MATLAB using custom software.

Whole-Genome Sequencing

Genomic DNA from consensus isolates was extracted via a PureLink Genomic DNA kit (Life Technologies), and prepared for sequencing using an Illumina Nextera XT kit at half volume, modified as in (*35*). The 20 end-point isolates from the four-step MEGA-plate as well as an ancestral sample were sequenced on an Illumina MiSeq. The 231 isolates from the intermediate-step MEGA-plate were sequenced on an Illumina HiSeq 2500. Reads were trimmed using Sickle (*36*) with parameters -q 20 -l 50, then aligned to Genbank (*37*) reference sequence U00096.2 with bowtie2 (*38*) using parameters --no-mixed --dovetail --very-sensitive --n-ceil 0,0.001, realigned with GATK 3.1-1 Indel Realigner with options -LOD 1.0 --entropyThreshold 0.10 and variants were called with GATK Haplotype Caller (*39*). Likelihood calls were normalized to the lowest non-mixed call and mixed calls were discarded. Mutations with likelihood at least 150 and at sites in which there were at least two discordant calls with minimum confidence 300 were taken as "real" SNPs or Indels. Mutations that appeared in the ancestral strain were discarded. Independent mutational events were inferred from cross-referencing the calls with the video-produced ancestries.

Calculation of dN/dS

The observed N (non-synonymous mutations) and S (synonymous mutations) were taken directly from sequencing. To calculate the expected N and S, all 5,979,186 possible SNPs in coding sequences in the reference genome were considered. Possible mutational events

were called as non-synonymous or synonymous based on the Genbank coding sequence annotations. From this, an expected N/S rate was calculated, assuming SNP events occur in the observed ratios. Dividing the empirical N/S by this corrected background N/S yields the reported dN/dS, i.e. the number of nonsynonymous mutations per nonsynonymous site divided by the number of synonymous mutations per synonymous site. The reported errors are the standard deviation of the Bayesian posterior estimate for the binomial distribution parameter for the observed nonsynonymous and synonymous substitutions, assuming a uniform prior, i.e.

$$std\big(\beta(N+1,S+1)\big)*(N_{obs}+S_{obs})*\frac{S_{tot}}{N_{tot}}$$

This can be seen as the error in the estimation of the binomial parameter that generated N_{obs} from N_{obs}+S_{obs} mutations, divided by the expected ratio.

figure S1

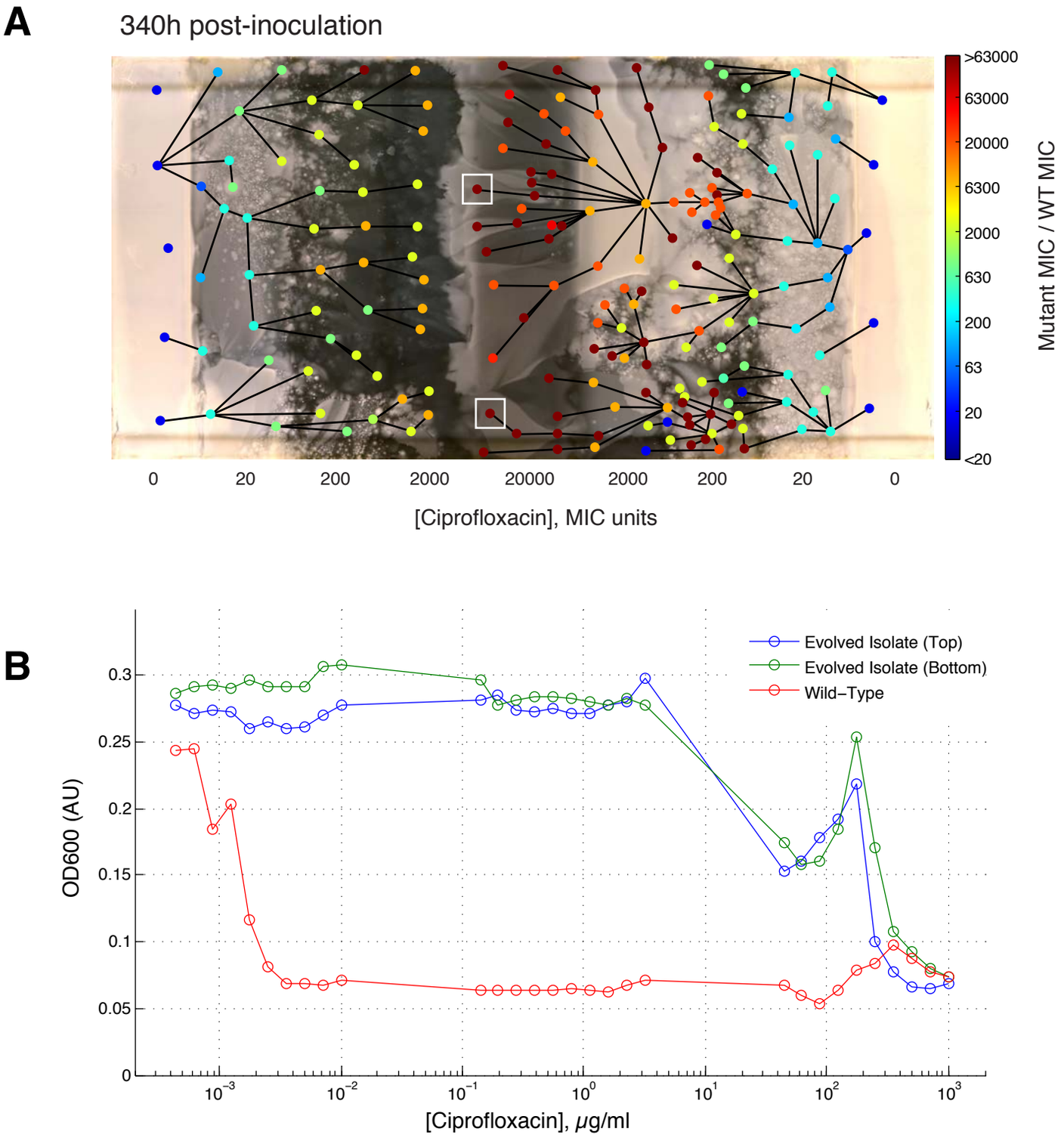

A
340h post-inoculation
>63000
63000
20000
6300
2000
630
200
63
20
<20
Mutant MIC / WT MIC
0
20
200
2000
20000
2000
200
20
0
[Ciprofloxacin], MIC units
B
Evolved Isolate (Top)
Evolved Isolate (Bottom)
Wild−Type
OD600 (AU)
0
0.05
0.1
0.15
0.2
0.25
0.3
10^−3
10^−2
10^−1
10^0
10^1
10^2
10^3
[Ciprofloxacin], μg/ml

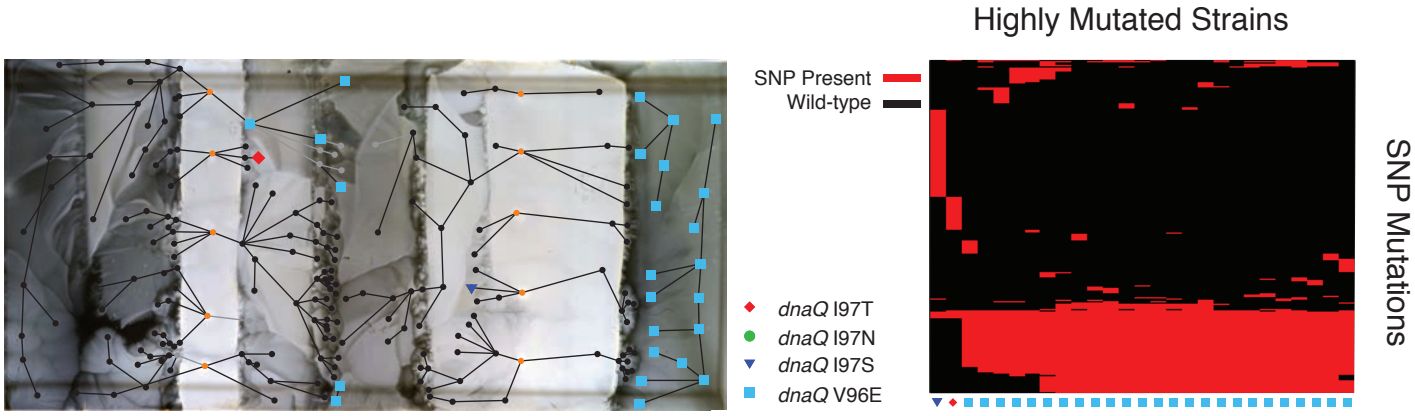
Fig. S1.

Measured MICs and locations of picked CPR Isolates. (A) Picked isolate locations on the 4-step MEGA-plate, colored by measured MIC**. (B)** Measured final density of ancestor and strains 182 and 194 grown in different CPR concentrations, showing over 100,000x increase in resistance.

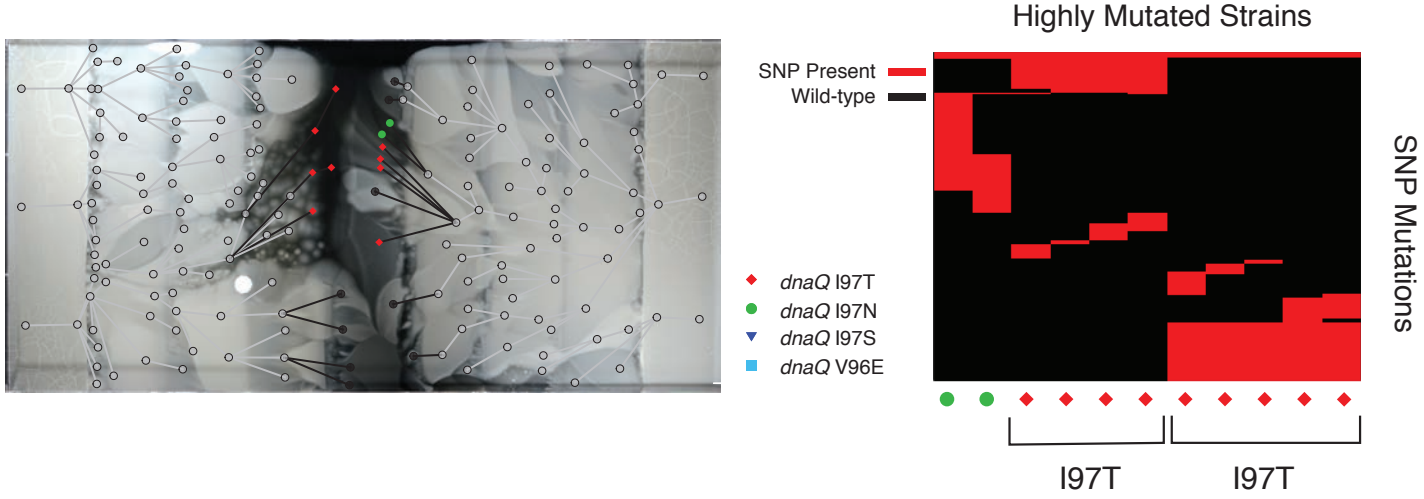
figure S2

A

Manifold highly mutated:

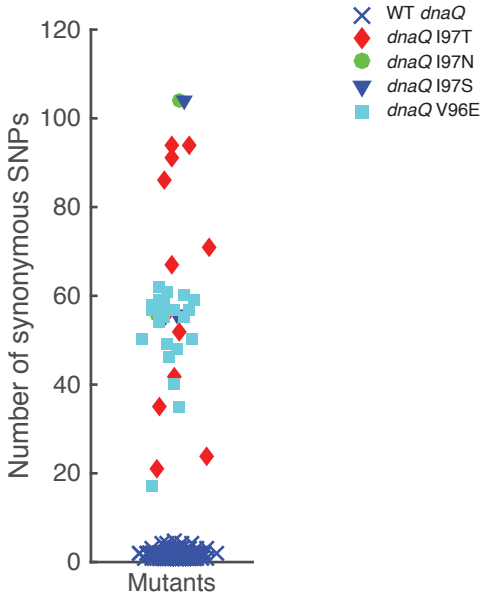


Symmetric highly mutated:



B

Synonymous SNPs



C

Rifampin disc-diffusion assay:

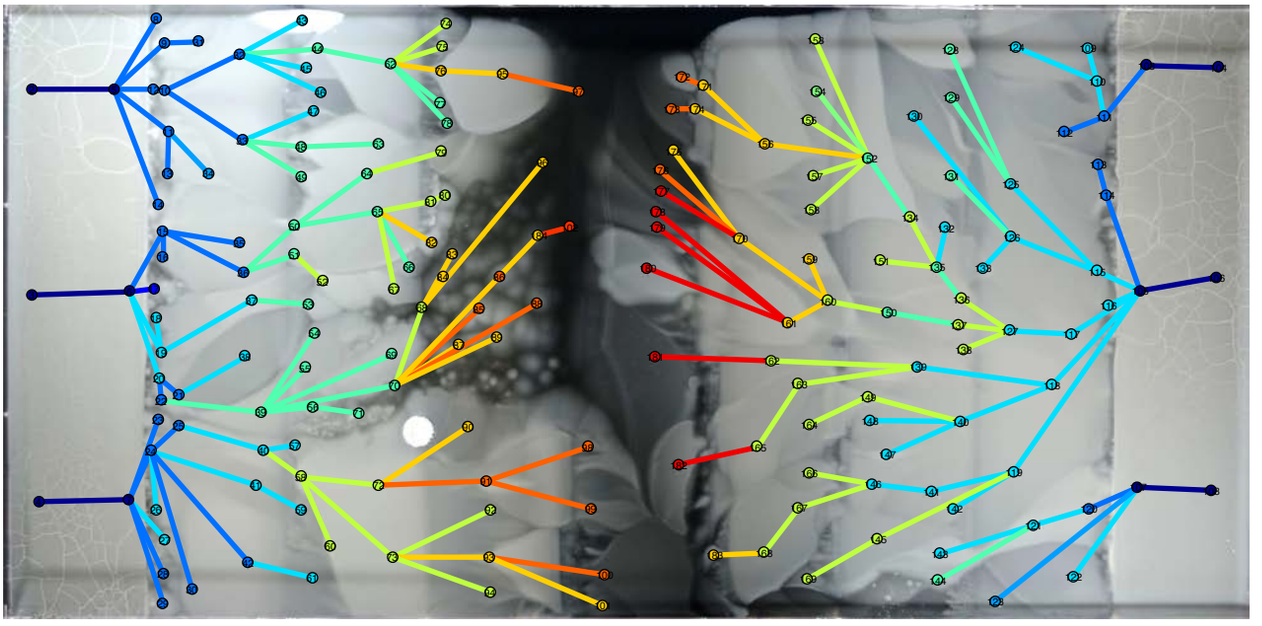
Strain	*dnaQ* Genotype	# of mutants
WT	WT	2
M82	WT	1
M169	WT	2
M186	WT	2
M74	I97T ◆	150
M77	I97N ■	26
M175	I97S ▼	23

Fig. S2

Mutators mutations appeared at least six times independently. (A) The locations and genotypes of all highly mutated isolates collected across both the 4-step and symmetric experiments. Shown on the right are clustergrams of the SNPs per strain across all marked isolates. The presence of clear blocks of mutated genes per group is indicative of independent emergence. (**B**) The number of observed synonymous SNP mutations between mutator and non-mutator phenotypes across all samples. All samples are marked and colored by the presence of a mutation in *dnaQ*, and horizontally jittered for clarity. (**C**) The number of mutations from a rifampin disc-diffusion assay for mutation rate in isolates carrying both the wild-type and putative mutator *dnaQ* alleles. The *dnaQ* mutants show an increased mutational rate.

figure S3

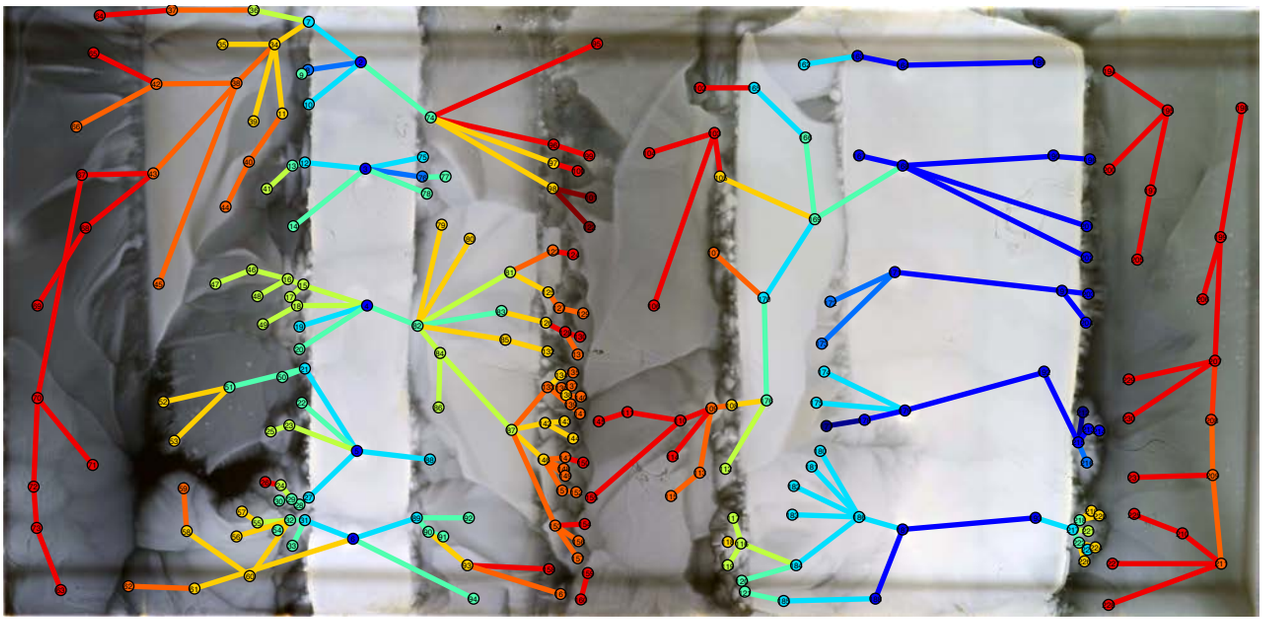
A



0 3 30 300 3000 300 30 3 0

[Trimethoprim], MIC units

B



3000 300 0 30 3000 3 0 0 3000

[Trimethoprim], MIC units

Fig. S3

Locations of picked isolates for cross-reference with the mutation charts. (A) Picked isolates from the 4-step trimethoprim MEGA-plate. Compensatory mutants are visible in the middle of the left-side 300x MIC band. **(B)** Picked isolates from the intermediate step trimethoprim MEGA-plate.

figure S4

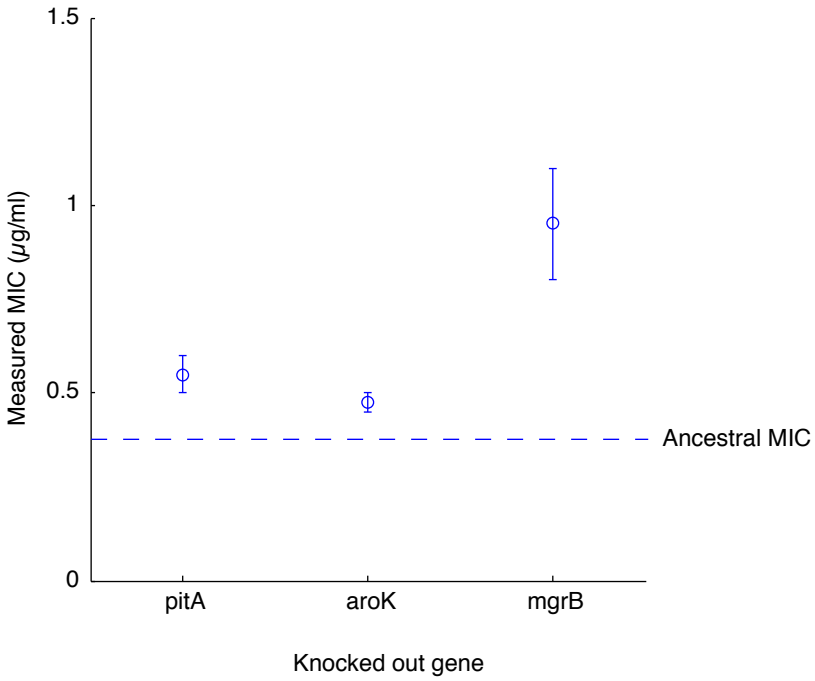

1.5
1
0.5
0
Measured MIC (μg/ml)
Ancestral MIC
pitA
aroK
mgrB
Knocked out gene

Fig. S4

Measured MICs of Keio mutants: MICs of three novel mutants from the Keio collection, with the lacA knockout (MEGA-plate ancestral) strain for reference. We also tested knockout mutants from the Keio collection (*34*) of the other genes that showed loss-of-function mutations: *marR*, *soxR*, *acrR*, *rng*, *tufA*, *deoB* and *marA* (data not shown). Of these genes, *deoB*, *acrR*, *rng* and *tufA* showed increase resistance. *marR*, *marA* and *soxR* did not show an increase.

figure S5

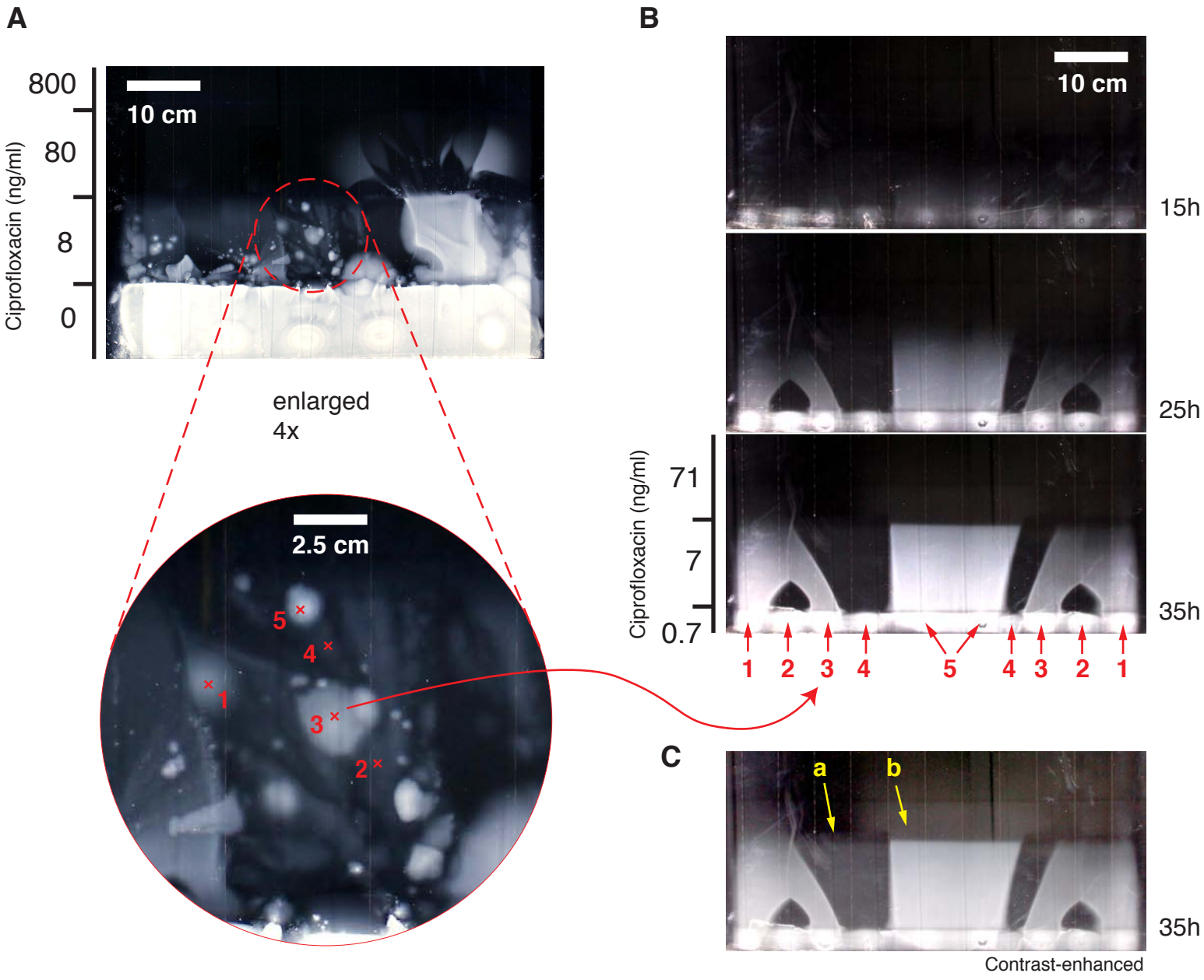
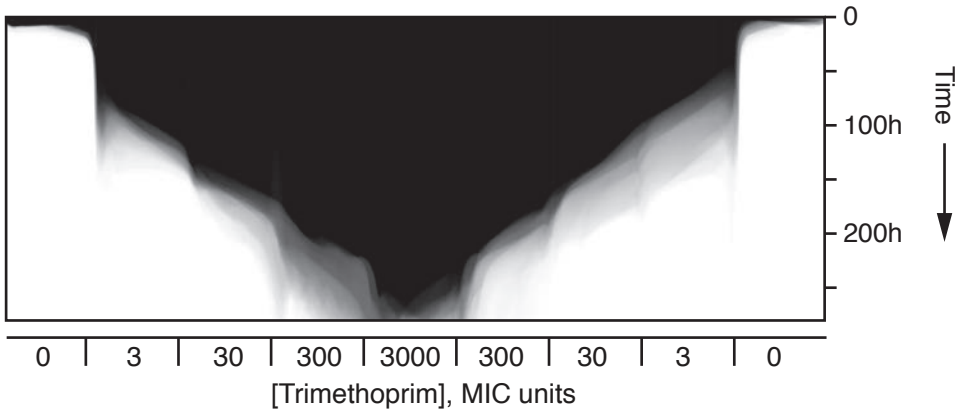

A
800
80
8
0
Ciprofloxacin (ng/ml)
10 cm
enlarged
4x
2.5 cm
5
4
1
3
2
B
10 cm
15h
25h
35h
71
7
0.7
Ciprofloxacin (ng/ml)
1 2 3 4 5 4 3 2 1
C
a
b
35h
Contrast-enhanced

Fig. S5

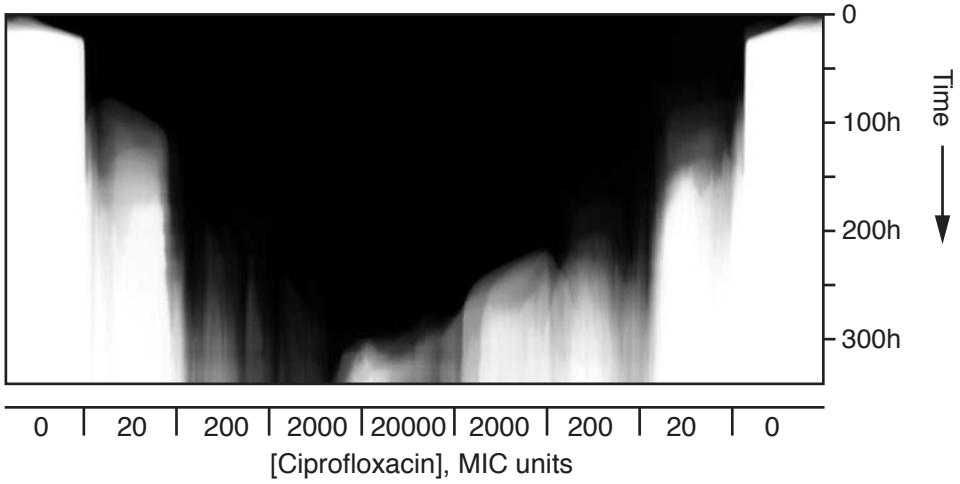
Trapped ciprofloxacin-resistant compensatory mutations are capable of spreading when not blocked by their ancestor. (**A**) Both low-density and compensatory were initially sampled from a Ciprofloxacin MEGA-plate. (**B**) The mutants were placed side-by-side, and both spread in the concentration at which they were picked. (**C**) Within 35 hours, the low-density mutant had stopped at the same boundary as in the previous experiment (a), while compensatory mutant 1 had overspread, albeit at lower density, into a higher concentration.

figure S6

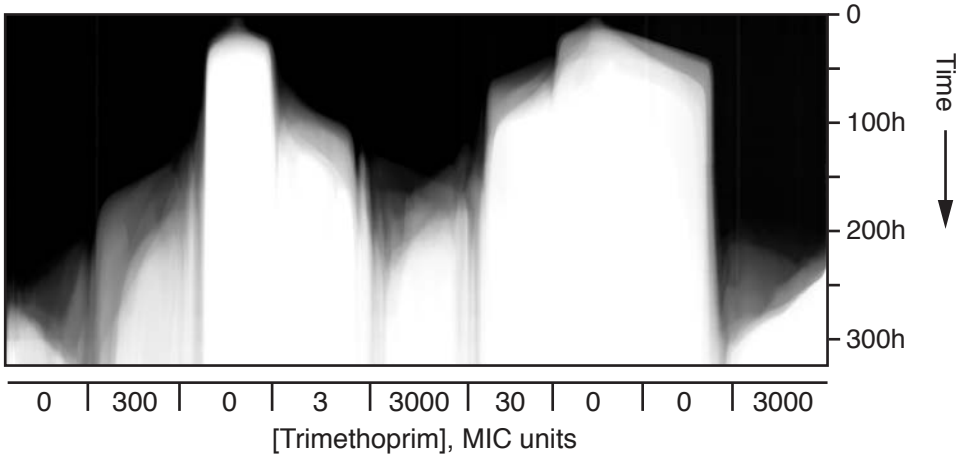
Symmetric Trimethoprim
(movie S1):



Symmetric Ciprofloxacin
(movie S3):



Manifold Trimethoprim
(movie S4):



Manifold Ciprofloxacin
(movie S5):

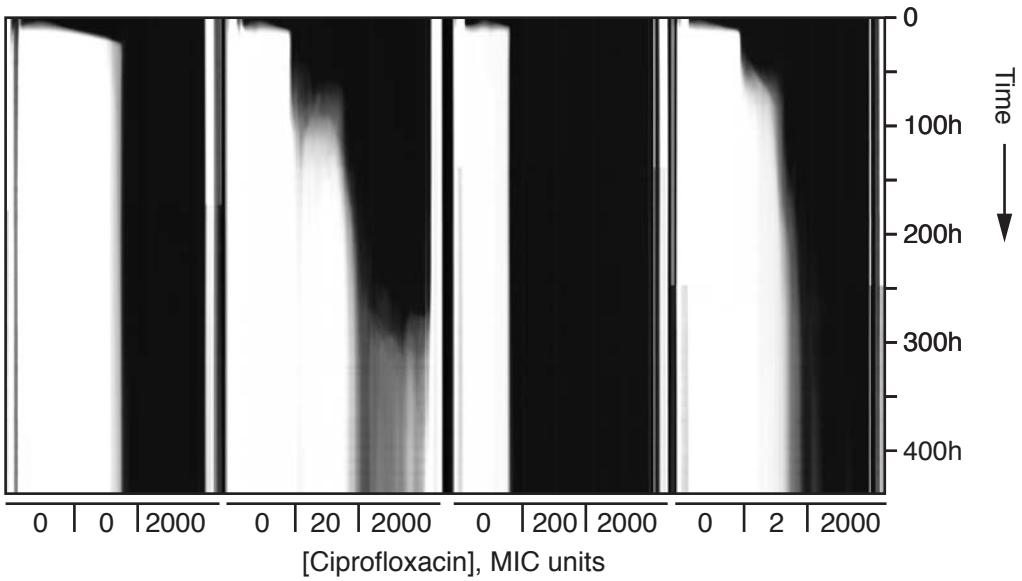


Fig. S6

Kymographs for the supplemental movies. For each horizontal band, the shaded color is the fraction of the corresponding vertical band of the plate that is at least 15% brightness. This thresholding allows the progress of multiple fronts to be visualized simultaneously.

Table S1.

All SNP and Indel calls from the four-step trimethoprim MEGA-plate samples. Sample names correspond with the numbers in fig. S3A.

Table S2.

All SNP and Indel calls from the intermediate step trimethoprim MEGA-plate samples. Sample names correspond with the numbers in fig. S3B.

Movie S1

Four-step trimethoprim MEGA-plate. The MEGA-plate with a trimethoprim gradient as in Fig. 1 (0-3-30-300-3000-300-30-3-0). Movie was compiled from time-lapse imagery every 10 minutes for 11.7 days, and played at 30fps (18000X speed). Condensation on the lid is visible in the first several frames, and a single contaminating colony appears on the plate.

Movie S2

Replicate four-step Trimethoprim MEGA-plate. The same setup as movie S1. Several contaminating strains are visible, but do not affect the overall evolutionary dynamics.

Movie S3

Four-step Ciprofloxacin MEGA-plate. The MEGA-plate with a CPR gradient as in fig. S1 (0-20-200-2000-20000-2000-200-20-0). Movie was compiled from time-lapse imagery every 10 minutes for 14.2 days, and played at 30fps (18000X speed).

Movie S4

Intermediate-step Trimethoprim MEGA-plate. The MEGA-plate with an intermediate-step TMP gradient (3000-300-0-30-3000-3-0-0-3000). Strips 3 and 7 were inoculated. Movie was compiled from time-lapse imagery every 10 minutes for 13.5 days, and played at 30fps (18000X speed). At 0:50 a mutator strain not evolved at the front can be seen invading the rightmost strip. Its identity as a mutator and descendent of mutators from the center strip was confirmed by sequencing.

Movie S5

Intermediate-step Ciprofloxacin MEGA-plates. Four small MEGA-plates, set up with CPR gradients 0-0-2000, 0-20-2000, 0-200-2000, and 0-2-2000 from left to right. Movie was compiled from time-lapse imagery every 10 minutes for 36.6 days, and played at 30fps (18000X speed).